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Original Article

Discovering potential bioactive compounds from Tualang honey

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ABSTRACT

Tualang honey is well known for its biological activities including as an antioxidant and anti-inflammatory. The compounds contributing to the biological activities are still unknown. Therefore, fractionation was carried out to prepare phenolic-rich extract from Tualang honey using ethyl acetate (EA). EA could recover a wide range of phenolic compounds obtained using column chromatography (CC) and liquid-liquid extraction (LLE) after acid hydrolysis. The yield of the EA fraction from LLE (0.83%) was higher than that from CC (0.39%). The LLE-EA fraction also contained more metabolites, especially organic acids (gluconic acid, succinic acid, hydroxybenzoic acid, hydroxydecanoic acid, abscisic acid, hydroxyoctanic acid), phenolic acids (caffeic acid, salicylic acid) and flavonoids (luteolin, hesperetin, kaempferol, apigenin, 3,7,4′-trihydroxyflavone, naringenin, chrysin, fisetin, vitexin, isoorientin and xanthohumol) as revealed in the liquid chromatography-mass spectrometry analysis. The recovered metabolites enhanced the radical scavenging capacity (free radicals and radical cations), reducing power and prostaglandin inhibition in cyclooxygenase assay. The enhancement was evident from the lower effective concentration of EA fractions than in crude honey and the alcohol-based fractions (methanol and butanol) which were used to remove the sugar components in honey.

Introduction

Honey has been widely consumed by humans since ancient times as a sweet food derived mostly from floral nectar by honey bees (*Apis* spp.) with many studies reporting that honey samples exhibited various bioactivities such as antioxidant, anti-inflammatory, antimicrobial, anticancer, anti-mutagenic, anti-diabetic and wound healing properties (Alvarez-Suarez et al., 2014; Rao et al., 2016; Melo da Cunha et al., 2018). The interesting pharmacological properties of honey are closely related to its phenolic compounds, particularly flavonoids and phenolic acids (Perna et al., 2013). These groups of compounds also vary according to the geographical and honey origin (Machado De-Melo et al., 2017).

Tualang honey is a multifloral honey produced by rock bees (*Apis dorsata*) which build their hives on the branches of large Tualang trees (*Koompassia excelsa)* which may grow up to tens of meters in height (Kishore et al., 2011). Sometimes, a giant Tualang tree may have more than 100 bee hives hanging on the branches (Ahmed and Othman, 2013). Tualang honey has less contamination from environmental factors, in addition to its numerous beneficial effects on human health and these benefits could be attributed to the presence of phenolic acids such as gallic, syringic, benzoic, cinnamic, coumaric and caffeic acids, as well as dominant flavonoids such as catechin, kaempferol, naringenin, luteolin and apigenin in the honey (Kishore et al., 2011; Campone et al., 2014; Chua and Adnan, 2014). However, scientific evidence on the contribution of phenolic compounds in enhancing the pharmacological properties of honey is very limited in the literature. Most studies have focused on crude honey samples for which the contribution of phenolic compounds could not be determined since the honey consists mostly of sugar.

In order to provide technical data on the contribution of phenolic compounds to the biological activities, the highly complex Tualang honey must be fractionated into phenolic rich extract by removing sugar components using techniques such as column chromatography (CC) and liquid-liquid extraction (LLE) which can be provided ethyl acetate (EA) honey fractions (Pascual-Maté et al., 2018). EA was used as the solvent of choice due to its efficiency in

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extracting a wide range of phenolic compounds (Pyrzynska and Biesaga, 2009). The performance of EA fractions was also compared with its crude honey in terms of antioxidant and anti-inflammatory activities using spectrophotometric assays.

Materials and methods

Honey samples

Tualang honey was purchased from the Federal Agricultural Marketing Authority (FAMA), Kuala Nerang, Kedah, Malaysia. Each honey sample was aliquoted into a 50 mL conical tube and stored in a refrigerator before use. The honey aliquot was homogenized by stirring with a glass rod before experiments.

Arachidonic acid (>95%), sulphanilamide (≥99%), celcoxib (≥98%), hematin, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (>98%, ABTS), 2,2-diphenyl-1-picrylhydrazyl (95%, DPPH), *N*,*N*,*N*′,*N*′-tetramethyl-p-phenylenediamine (99%, TMPD) and cyclooxygenase 2 (COX-2, ≥8000 units/mg protein) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ascorbic acid, sodium nitroprusside dihydrate and N-1-napthylethylenediamine dihydrochloride were bought from Bio Basic (Markham, Ontario, Canada). Iron (II) sulphate (FeSO4), dimethyl sulfoxide (DMSO), ethyl acetate, formic acid and phosphoric acid (H_3PO_4) were sourced from Merck (Darmstadt, Germany). Hydrochloric acid (HCl) and phosphate saline buffer were obtained from J.T. Baker (Waltham, MA, USA) and Gibco (New York, NY, USA), respectively. Potassium persulphate, ferric chloride ($FeCl_3$) and analytical grade solvents such as butanol and methanol were purchased from Qrec (Selangor, Malaysia).

Column chromatography of honey

Honey sample (5 g) was hydrolyzed using acidified methanol (pH 2.0) with continuous stirring for 30 min at room temperature. The hydrolyzed honey was loaded onto a packed column with dimension of 2 × 30 cm (diameter × height). The glass column was packed with silica gel 60 with a particle size of 70–230 mesh (Macherey-Nagel; Duren, Germany). Different polarities of solvents; ethyl acetate (polarity index, P = 4.4) and methanol (P = 5.1) were used to fractionate the honey. A 200 mL sample of each solvent (about 2 column volumes) was used for each elution. The collected fraction was dried by a rotary evaporator at 50 °C.

Liquid-liquid extraction of honey

Liquid-liquid extraction was carried out to prepare each EA fraction of Tualang honey according to the procedures described by Spilioti et al. (2014) with some modifications. Each honey sample (5 g) was weighed and digested with 4 mL acidified water (pH 2.0) by vigorous stirring for 10 min. Then, 4 mL butanol was added to the honey solution and vortexed for 10 min, followed by centrifugation at 5000 rpm. The mixture was separated into two immiscible phases. The top layer of butanol was collected. Another 4 mL of butanol was added into the remaining aqueous phase to repeat the extraction. Butanol extract was collected and combined for vacuum drying. The remaining aqueous layer was then discarded. The yellowish butanol residue was washed twice with EA (5 mL). The collected EA fraction was dried and weighed for the determination of extraction yield.

Radical scavenging capacity of honey fractions

2,2-diphenyl-2-picrylhydrazyl assay

The 2,2-diphenyl-2-picrylhydrazyl (DPPH) assay was performed according to the method described by Chua et al. (2013) with some minor modifications. Honey samples were completely dissolved in methanol and prepared in different concentrations ranging from 0 to 200 mg/mL. The $DPPH^{\bullet}$ solution was prepared by dissolving 2 mg DPPH in 100 mL of methanol. Honey solution (0.75 mL) was added with 1.5 mL of the $DPPH^{\bullet}$ solution and incubated for 15 min in a dark place at 25 °C. The absorbance of the mixture was measured using an ultraviolet-visible spectrum (UV-Vis) spectrophotometer at 517 nm. Ascorbic acid was used as the standard chemical for calibration curve construction. All experiments were performed in triplicate. The capacity of honey fractions to scavenge free radicals was determined and the results we expressed as the effective concentration to achieve 50% inhibition (EC_{50}).

2,2′Azinobis(3-ethylbenzothiozoline-6-sulfonic acid) disodium salt radical assay

The antioxidant capacity of honey fractions was also examined based on their scavenging activity against radical cations. 2,2′Azinobis (3-ethylbenzothiozoline-6-sulfonic acid) disodium salt radical (ABTS) assay was performed according to the method described by Biskup et al. (2013) with some modifications. The $ABTS^{\bullet+}$ solution was prepared by reacting ABTS (7 mM) with potassium persulfate (2.45 mM) at a ratio of 1:1, and then incubating overnight in a dark place. The solution was diluted with distilled water to have an absorbance of 1.00 at 734 nm. Honey fractions were also dissolved in distilled water to prepare honey solution at concentrations ranging from 0 to 1000 mg/mL. Then, 2 mL diluted $ABTS^{\bullet+}$ was added to 100 μL honey solution, and incubated under subdued light conditions for 6 min. The absorbance was measured at 734 nm using a UV-Vis spectrophotometer.

Griess assay

Another assay to evaluate the scavenging capacity of honey fractions was based on the inhibition of free radical nitric oxide (•NO) which can be measured based on the formation of its breakdown product, nitrite (NO_2^-). Since •NO is also a cellular signaling molecule, this Griess assay is also widely used to investigate inflammation in cell-based experiments. Griess assay was carried out according to the procedures reported by Sannigrahi et al. (2010) and Abdelwahab et al. (2011) with some modifications. Griess reagent was prepared by mixing 1% sulfanilamide (25 mL) and 0.1% naphthylethyenediamine dihydrochloride (25 mL) in 2.5% phosphoric acid (H_3PO_4). Sodium nitroprusside (10 mM) was prepared in phosphate saline buffer. Honey fractions were dissolved in dimethyl sulfoxide (DMSO) to prepare a series of honey solutions with concentrations ranging from 0 mg/mL to 30 mg/mL. A 50 μL honey solution was added into 0.2 mL sodium nitroprusside and incubated in a 96-well plate for 2 h at 25 °C. Then, 50 μL of the mixture was taken and added into 100 μL Griess reagent for further incubation in the dark for another 10 min. The absorbance was recorded after incubation using an enzyme-linked immunosorbent assay plate reader at 546 nm.

Reducing power of honey fractions

The reducing power of honey fractions was determined using ferric reducing antioxidant power (FRAP) assay which was carried out according to the procedures reported by Chua et al. (2013) with modification. FRAP reagent was freshly prepared by mixing 2.5 mL 2,4,6-tripydyl-*s*-triazine complex (10 mM, Fe^{3+}-TPTZ) in hydrochloric acid (40 mM), 2.5 mL iron (III) chloride (20 mM, $FeCl_3$) and 25 mL acetate buffer (0.3 M, pH 3.6). The reagent solution was kept in the dark at 37 °C before use. Honey fractions (50 mg/mL) were prepared in DMSO. Each sample (0.2 mL) was mixed with 1.8 mL FRAP reagent, and incubated at room temperature under subdued light conditions for 10 min. The absorbance was measured at 593 nm using a UV-Vis spectrophotometer. Ascorbic acid (10 mg/L)

was used as a positive control. FRAP values were estimated from the standard curve of iron (II) sulphate ($FeSO_4$) with the concentrations in the range 0–1000 μM and expressed as micromolar Fe^{2+} equivalent per 100 g honey fraction.

Cyclooxygenase assay

Cyclooxygenase assay was performed according to the procedures described by Copeland et al. (1994), and Petrovic and Murray (2010) with some modifications. A 106 μL Tris-HCl buffer consisting of Tris-buffer solution (100 mM) and hematin (3 μM) was placed into a 96-well plate. Then, 40 μL honey fractions with concentrations in the range 0–30 mg/mL and 40 μL COX-2 (40 units) were added into the buffer solution. The mixture was incubated for 15 min at ambient temperature in a dark place. A 10 μL sample of TMPD (20 mM) and 4 μL arachidonic acid (10 μM) were immediately added into the mixture to initiate the synthesis of prostaglandin. The formed intermediate peroxide product (prostaglandin G2) oxidized TMPD into a purple-colored TMPD complex. The absorbance was spectrophotometrically measured at 611 nm after 15 min of incubation under subdued light conditions. Celecoxibs was used as the standard chemical.

Liquid chromatography-mass spectrometry analysis

A hyphenated analytical technique of Liquid chromatography-mass spectrometry (LC-MS/MS) was used for compound separation and identification. The liquid chromatography (Ultimate 3000; Dionex Corporation; Sunnyvale, CA, USA) system was coupled with a diode array detector (Dionex Ultimate 3000) from the same company and a quadrupole and time-of-flight mass spectrometer (QSTAR Elite; AB Sciex; Foster City, CA, USA). A C18 XSelect HSS T3 column (2.1 mm × 100 mm, 2.5 μm) was used for compound separation at a flow rate of 200 μL/min. The binary solvent system consisting of solvent A (water with 0.1% formic acid) and solvent B (acetonitrile) flowed at the following gradient: 0–10 min, 10% B; 10–20 min, 10–80% B; 20–25 min, 80% B; 25–25.1 min, 80–10% B; and 25.1–30 min, 10% B. The injection volume was 5 μL. Each sample was scanned at 254 nm. All samples were filtered through a 0.2 μm nylon membrane filter prior to injection.

The quadrupole time-of-flight mass spectrometer was used for compound identification based on the detection of characteristic fragment ions matched to literature data and the online database (European MassBank, 2011). The masses of compounds were screened in the range 100–1000 m/z. Nitrogen gas was used for nebulizing (at 40 pounds per square inch; psi) and as a curtain gas (25 psi). Collision gas was set at 3, the accumulation time was 1 s for TOF MS and 2 s for each product ion scan. The voltage of the ion spray was 5500 V for the positive ion mode and −4500 V for the negative ion mode. The declustering potential was 40 V and the focusing potential was set at 200 V.

Results and discussion

Fractionation of honey

Tualang honey was fractionated to remove the most abundant component (sugar) prior to bioactivity investigation. The sugar component (mostly monosaccharides such as glucose and fructose) accounts for at least 65% of the honey content (Moniruzzaman et al., 2013). Therefore, it is important to remove sugar from honey to avoid matrix interference during analysis, and to concentrate the bioactive compound(s) for bioassays. The presence of sugar may explain the inconsistent results reported by previous investigators, even though they used the same type of honey (Campone et al., 2014). Nevertheless, honey is a natural product which is subjected to great variance because of climatic and geographical factors, in addition to diverse honey origins. This has limited the studies to produce conclusive data on the bioactive compounds of honey samples.

CC of Tualang honey was carried out based on the principle of normal phase chromatography. Polar absorbent, silica gel 60 was used to pack the column for honey fractionation using eluents such as less polar EA to more polar methanol. The yield of EA fraction from LLE (0.83 ± 0.15%) was lower than methanol fraction (76.80 ± 13.18%) because the methanol fraction mostly contained the sugar components of honey. It is also important to note that the yield of the EA fraction from LLE was twice as high as the result from CC (0.39 ± 0.09%). The yield difference between the methods of LLE and CC was significant based on a Student's *t*-test with a two-tailed distribution ($p < 0.05$). Some compounds might have been eluted into the methanol fraction together with sugar during CC. This would also explain the high yield of the methanol fraction in CC. Therefore, the yield of the EA fraction from CC was slightly lower than from LLE in this study.

Bioactivities of honey and its fractions

Table 1 clearly indicates that fractionation after acid hydrolysis can improve the bioactivities of honey to scavenge free radicals and to inhibit inflammation. The improvement can be seen from the reduction of EC_{50} from crude honey to fractionated honey, particularly for the EA fractions. The scavenging capacity of the methanol fraction (ME) increased approximately 2–9 times that of its crude honey. It was observed that mostly the sugar component of the honey was recovered in the sticky ME. Interestingly, EA fractions showed 35 to 160 times higher scavenging capacity than for crude honey. The EA fraction of LLE appeared to have more radical scavengers because of its lower EC_{50} than the EA fraction of CC.

The linear relationship of polyphenols and DPPH• radical scavengers was proven by Khalil et al. (2012) who reported that the higher the polyphenolic content in a honey sample, the higher the DPPH• free radical scavenging activity that would be obtained. The final products of this scavenging reaction is a phenolic acid quinone (Tsimogiannis and Oreopoulou, 2006). Phenolic compounds are able to scavenge free radicals, mainly due to the presence of hydroxyl groups in their chemical structures (Rice-Evans et al., 1996; Biskup et al., 2013). However, the scavenged number of DPPH• varies according to the chemical properties, including the availability of hydrogen donation (Ajila et al., 2007). The readiness of hydrogen donation may be affected by the steric hindrance of the carboxyl group which is located next to the hydroxyl group (Shahidi and Chandrasekara, 2010). Hence, the number and position of hydroxyl groups in the skeletons of the phenolic compounds are very important to the scavenging capacity of antioxidants (Nenadis et al., 2004). This had been proven by Chen and Ho (1997) whose comparison found that caffeic acid appeared to be higher in scavenging capacity than its counterparts such as ferulic acid and coumaric acid. The factor most influencing high scavenging capacity still remains unclear.

It is also noteworthy that the sample needs to be dissolved in an alcohol-based medium as the scavenging activity of flavonoids and phenolic acids could perform better than in non-alcohol solvents such as ethyl acetate and chloroform (Tsimogiannis and Oreopoulou, 2006). Therefore, DPPH assay is more suitable for hydrophilic compounds. On the other hand, ABTS assay was reported to be more versatile for both hydrophilic and lipophilic compounds; ABTS assay has less interference on the spectrum since absorbance was measured at a higher range of visible frequency at 760 nm (Re et al., 1999; Prior et al., 2005; Perna et al., 2013). Based

Table 1
Scavenging and anti-inflammatory activities of honey and its fractions.

Honey	Bioassay (EC_{50}; mg/mL)				FRAP (μmol Fe^{2+}/100 g)
	DPPH	ABTS	Griess	COX-2	
Crude honey	80.186	308.793	942.945	108.500	28.745
CC-ME fraction	40.193	33.864	222.540	247.762	52.512
CC-EA fraction	2.244	4.980	8.217	1.377	756.743
LLE-EA fraction	1.360	1.926	3.280	4.517	725.342
Ascorbic acid	0.004	0.006	0.202	0.013[a]	957.401[b]

Note. CC = column chromatography, LLE = liquid-liquid extraction, ME = methanol, EA = ethyl acetate.
[a] Celecoxib.
[b] Ascorbic acid at 10 mg/L.

on the characteristics of both assays, the CC-EA fraction might contain equal amounts of hydrophilic and lipophilic compounds, whereas the LLE-EA fraction might have more hydrophilic compounds. The EC_{50} of the CC-EA fraction in the ABTS assay was about twice as high as the value in the DPPH assay, while the LLE-EA fraction produced almost similar EC_{50} values in both DPPH and ABTS assays. Somehow, the scavenging capacity of EA fractions was still a few hundred-folds lower than ascorbic acid which is also a well-known antioxidant compound.

Similarly, the technique of acidic hydrolysis followed by fractionation also improved the reducing power of honey, especially the EA fractions (Table 1). Crude honey had the lowest ferric reducing power compared to its ME and EA fractions at the same concentration (50 mg/mL). Polyphenols in both EA fractions are likely to be potent reductants by donating electrons to ferric ions. The FRAP values of both the ME and EA fractions also indicated that protons from polyphenols are easier to be donated than protons from sugar moiety. Somehow, the reducing power of EA fractions was still far lower than ascorbic acid which could achieve 957.40 μmol Fe^{2+}/100 g at a concentration of 10 mg/L.

The Griess and COX-2 assays revealed that EA fractions might contain potent polyphenols as anti-inflammatory agents. The results in Table 1 also indicate that sugar moiety is unlikely to be a good anti-inflammatory agent because of higher EC_{50} for the ME fraction than its crude honey. However, polyphenols in the EA fractions could scavenge •NO into NO_2^- and inhibit oxidization of arachidonic acid into prostaglandin effectively. The LLE-EA fraction had a higher radical scavenging capacity, but its anti-inflammatory ability was slightly lower than for the CC-EA fraction. Reactive oxygen species (ROS) are usually generated during metabolism, and their production would be enhanced in the presence of radicals from external stimuli (Schieber and Chandel, 2014). ROS and radicals trigger the expression of NF-κB genes and further induce isozyme of COX 2 during inflammation (Chen et al., 2005). Therefore, polyphenols play their roles to inhibit the reaction chain of ROS before leading to a series of transduction pathways which will eventually lead to inflammatory cascades (Yumi and Richard, 2001).

Liquid chromatography-mass spectrometry analysis

Both EA fractions from CC and LLE were analyzed using LC-MS/MS. The chromatograms of both EA fractions are presented in Fig. 1 which clearly indicates that more peaks were detected in the LLE-EA fraction than the CC-EA fraction. The CC-EA fraction shows an almost similar chemical profile for polar and semi-polar compounds with the LLE EA fraction. However, most of relatively fewer polar compounds could only be detected in the LLE-EA fraction as fewer polar compounds were eluted from the LC column and

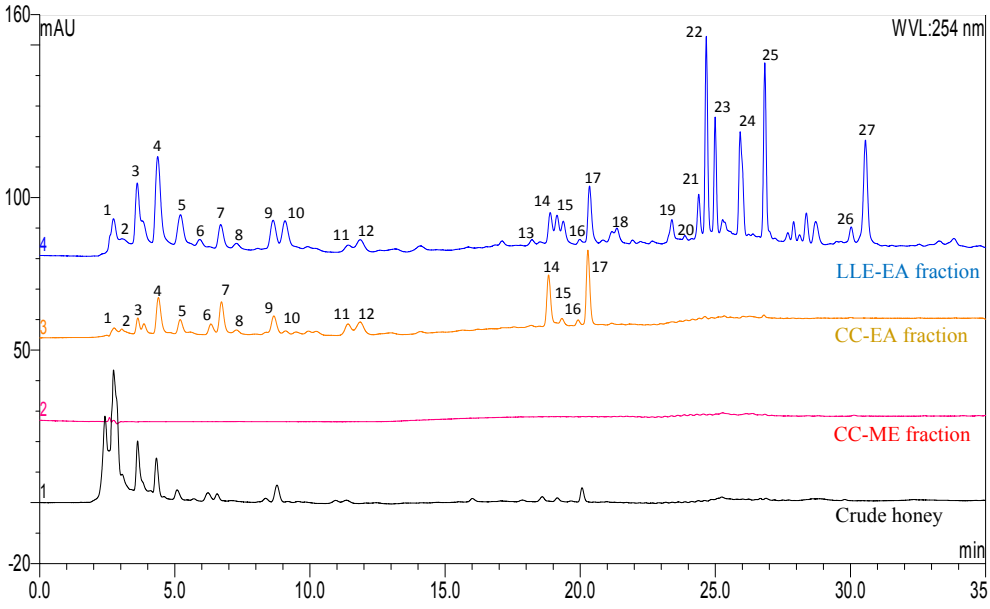

Fig. 1. Chromatograms of Tualang honey ethyl acetate (EA) fractions prepared from column chromatography (CC) and liquid-liquid extraction (LLE) compared to its methanol (ME) fraction and crude honey; 1. Glucose, 2. Gluconic acid, 3. Succinic acid, 4. Caffeic acid, 5. Luteolin, 6. Hesperetin, 7. Salicylic acid, 8. Hydroxybenzoic acid, 9. Kaempferol, 10. Pyridoxamine, 11. Apigenin, 12. 3,7,4′-trihydroxyflavone, 13. Naringenin, 14. Chrysin, 15. Fisetin, 16. Hydroxydecanoic acid, 17. Abscisic acid, 18. Orotic acid, 19. Hydroxyoctanic acid, 20. Pyridoxal, 21. 6-hydroxy-4-methylcoumarin, 22. Lauryl diethanolamide, 23. 2-amino-1,3-dihydroxyactadecane, 24. Vitexin, 25. Linolenic acid, 26. Isoorientin, 27. Xanthohumol.

detected at the end of the chromatogram. The use of butanol was effective in extracting a wider range of compounds from honey, and subsequently the compounds were recovered by partitioning them into the relatively less polar solvent, EA during LLE. Some of the sugars which were extracted by the butanol would remain in the butanol. This would explain why the remaining butanol did not show any compound detected in the chromatogram at 254 nm (data not shown). Sugar will be only detected at a lower wavelength (below 200 nm). This explanation also applied to the ME fraction of CC which showed no peak at 254 nm.

A mass analyzer was used to ionize and fragment the compounds. The fragment ions were matched to the literature data and online database for compound identification based on the detection of characteristic fragment ions. It was found that more flavonoids, phenolic acids, organic acid and other hydrocarbons were detected in the LLE-EA fraction. This would also explain the higher yield and stronger antioxidant capacity in the LLE-EA fraction than the CC-EA fraction and is consistent with the findings of Kassim et al. (2010) who reported that EA fractions of Gelam honey were found to have more polyphenols.

Tualang honey is known for its pharmacological activities in recent studies. The technique of fractionation after hydrolysis on honey samples by CC and LLE seemed to improve the antioxidant capacity and anti-inflammatory activity substantially. This suggested that the biological activities of honey are mainly attributed to the presence of phytochemicals collected by honey bees from flower nectar or other plant-based materials. Most of the phytochemicals such as phenolic acids, organic acids and flavonoids could be extracted by EA and detected in the EA fractions. However, methanol and butanol showed a sticky appearance which could have been due to the large amount of sugar in those fractions. Therefore, EA fractions either from CC or LLE enhanced the biological activities compared to crude Tualang honey. The technique of LLE could prepare a higher yield of the EA fraction and with better antioxidant capacity because more phytochemicals could be recovered from the honey.

Conflict of interest

The authors declare that they have no conflict of interest.

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